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13. ABSTRACT (Maximum 200 Words)

Prostate cancer is the second leading cause of death among American men. The present proposal extends the molecular progress in prostate cancer into clinical practice by developing innovative markers. The current androgen ablation treatment is initially therapeutic, but most patients relapse. A novel androgen substitution approach uses androgens to promote terminal differentiation and arrest cancer. Markers of differentiation, however, are not known in the prostate. We discovered a novel gene, AS3 (new name APRIN), a marker of proliferative arrest and differentiation. We mapped the AS3 gene on chromosome 13 and we found that the loss of the gene correlates with a high incidence of prostate cancer. In many of the cancers genetic markers are not informative, raising the possibility that epigenetic mechanisms contribute to AS3 silencing. The objective of this proposal is to investigate the epigenetic mechanisms to assess AS3 gene silencing in prostate cancer. In the first year we established the technology to isolate cancer specific DNA and worked out the methylation specific PCR and sequencing techniques on the AS3 promoter. We generated the first data that methylation of the APRIN promoter is critical in the silencing of the gene and correlates with hormone resistance in the LNCaP-TJA prostate cancer cell line. In the second year we analyzed DNA methylation in the entire CpG island of the APRIN promoter. We identified the methylation map and discovered methylation hot spots. By using these data we analyzed APRIN promoter methylation in various prostate cancer samples and established preliminary correlations with histology.

14. SUBJECT TERMS
APRIN gene, proliferative arrest, epigenetic silencing, promoter methylation, prognostic marker, prostate cancer

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INTRODUCTION

The statistics published every year by the American Cancer Society indicate that prostate cancer continues to increase in the United States. We know more about the molecular processes than ever before, but the application of this knowledge in the clinical care is practically non-existent.

The goal of this proposal is to establish a technology which will allow highly sophisticated molecular information to be directly applied into clinical practice. The methods we propose can detect gene silencing using a novel approach at the level of the promoter. When the gene is silenced, it is blocked by methylation and we can detect this modification (hypermethylation).

The gene we target and the methods we propose may be able to have a significant impact on the diagnosis and therapy of prostate cancer. Our previous work has introduced novel findings into this field of prostate cancer research. We investigated the mechanisms of terminal differentiation in the prostate and identified a key regulator of the process, the AS3 (new name APRIN) gene (1, 2, 3). Expression of the gene indicates differentiation in animals and brings about proliferative arrest in cell culture (4). We also found that AS3 is frequently lost in prostate and other cancers (5), strongly suggesting that it is also a tumor suppressor.

We hypothesize that the status of AS3 is critical in the prognosis, the therapy and the outcome of prostate cancer. The presence of AS3 indicates that differentiation is functional in these cancers and that hormonal therapy will benefit the patients. The information, therefore, on the status of AS3 may be the most important variable to make therapy decisions.

The studies we proposed will supply that information in an extremely sensitive, specific and simple way. A line of evidence indicates that APRIN is silenced in many cancers (5, 6, 7, 8). We investigate if the silencing is the result of DNA methylation in the promoter. The methods are based on the amplification of modified DNA which reflects the methylation status (9, 10, 11). Since the modification we test (APRIN promoter methylation) is absent from normal cells, any positive signal indicates disregulation and may serve as the earliest sign of malignant cells in the body.

In the first year of the project we established the basic reagents to perform further clinical studies. Using this new set of reagents we generated the first data that DNA methylation takes place in the APRIN promoter and established a preliminary correlation with promoter methylation and APRIN silencing.

In the second year of the project we completed several research goals we initiated in the first year. We analyzed DNA methylation in the entire CpG island of the APRIN promoter, about 800 base pairs. We identified the methylation map, methylation densities and discovered methylation hot spots. These new data allowed us to analyze APRIN promoter methylation in various prostate cancer samples. We found that in spite of the low DNA quality, we could detect APRIN methylation silencing in most of the archived samples. Since our access for clinical samples was limited in the second year, this goal will be performed in the third year, an extension we have received from the DOD.

RESULTS

TASK 1: *(i) To establish the methodology to isolate DNA from various biological sources and (ii) to use the isolated DNA to evaluate methylation-specific techniques on the AS3 gene. (These activities are expected to continue to a lesser extent during the second year, depending on the availability of the clinical samples and archival sources) (Months 1-12)*

We followed the reviewer's advice in his evaluation of our Progress Report for the first year and we completed several goals that we initiated in the first part of the project. We worked out various DNA extraction and purification methods in the first year and established the technology for promoter methylation analysis in the promoter of a new gene, APRIN.

APRIN is involved in differentiation and hormonally regulated proliferative arrest in reproductive tissues and in breast, prostate and other cancers. Microsatellite marker studies indicated genomic losses in APRIN in about 20 % of these cancers. Microarray analyses, SAGE data and our immunohistochemical studies showed, however, that APRIN is downregulated or silenced in about 70-90% of cancers. To search for the mechanisms, we found a highly CG-rich CpG island in the promoter and around the first exon of APRIN on chromosome 13 (Figure 1). CpG islands are known to be silenced by DNA methylation, so the finding raised the possibility that in the majority of cancers APRIN is silenced by epigenetic mechanisms, by promoter methylation.
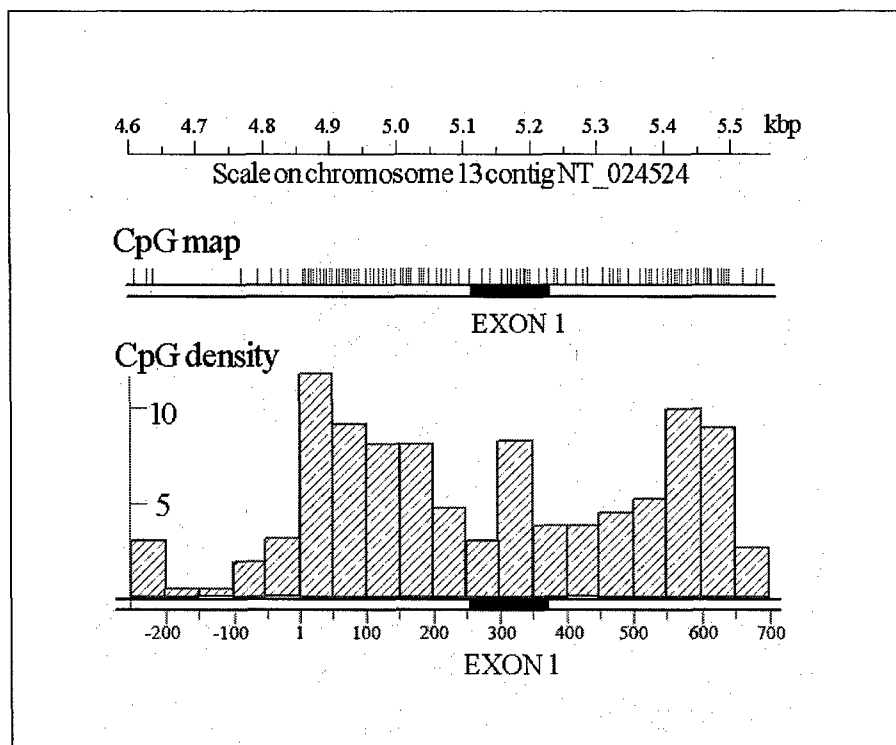
Figure 1   The CpG island of the APRIN (AS3) promoter. The vertical red lines in the CpG map indicate CpG units. Exon 1 of the APRIN gene is shown in blue. The CpG density is expressed in percentage (scale at the left). The graph depicts CpG densities in 50 basepair units. The horizontal numbering in the bottom panel starts with the first base of the CpG island and corresponds with the numbering in Figure 2 and with primer and CpG numbers throughout the report.

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As a reference for the following sections, **Figure 2** below shows the APRIN CpG island sequence and some of the primers we used.

```
  1 CGCGGGCCCCGCGGCGGCGTGCCCGTGTGGTTGCGTGCGCGCGGACGGGGCGGGGCTGAG
    ++++||:::++++|++|++||::++|||||||||++||++++++||++|||++|||:||||
  1 CGCGGGTTTCGCGGCGGCGTGTTCGTGTGGTTGCGTGCGCGCGGACGGGGCGGGGTTGAG
   <- MTn38                                                 MC50
 61 GAGGGACGTGCGCGCGCGCGGACGGGGCGGGGTGGAGCTCGGAGGGCGGACGCGCTCGGG
    ||||||++|||++++++++++|||++|||++||||||||:|++||||||++|++++:|++||
 61 GAGGGACGTGCGCGCGCGCGGACGGGGCGGGGTGGAGTTCGGAGGGCGGACGCGTTCGGG

121 GCCGTCTGGTCGGCAGGGGGCGCTGCGCACCCATGCCGCGCTCTGCCTGCCCCCGGGTGC
    |:++|:||||++|:||||||++:||++:|:::|||:++++:|:||::||:::++|||:
121 GTCGTTTGGTCGGTAGGGGGCGTTGCGTATTTATGTCGCGTTTTGTTTGTTTCGGGTGT
                        MC133
181 TGTCCGCGGCGGTTCGATTGGAGCAGCCGCCGAAGAGCGCTTGGCGCCATTTTGAAGCGG
    |||:++++|++|||++|||||||:||:++:++|||||++:||||++::||||||||||++|
181 TGTTCGCGGCGGTTCGATTGGAGTAGTCGTCGAAGAGCGTTTGGCGTTATTTTGAAGCGG
                                                            MC229

241 AGAGGAGGAGGAACGGCAGCGCTGGCTGCGGAAGGGGAGGGGGGGGGAGAAGGCGATTGG
    ||||||||||||||++|:|||:||||:||++|||||||||||||||||||||||++|||||
241 AGAGGAGGAGGAACGGTAGCGTTGGTTGCGGAAGGGGAGGGGGGGGGAGAAGGCGATTGG
```

E X O N 1

```
301 ATGCGGCGGCGGCGGCGGATCCCGGAGAGCCCCGGAGTGAGCGGAGTAGCGAGTCGGCAA
    |||++|++|++|++|++|||::++|||||:::++|||||||++|||||++|||++|:||
301 ATGCGGCGGCGGCGGCGGATTTCGGAGAGTTTCGGAGTGAGCGGAGTAGCGAGTCGGTAA

361 CCCGGAGGTAGGAAACAATTCAGTTAACGGAAAGAAAATGTCTCTCTTCTCTGGCTGCGG
    ::++||||||||||||:||||:||||||++||||||||||||:|:|:||:|:|||:||++|
361 TTCGGAGGTAGGAAATAATTTAGTTAACGGAAAGAAAATGTTTTTTTTTTTTGGTTGCGG
             MT400
```

```
421 CGCTCCATTTTTCAGCGGCCGCCACTGCCGCTGTGTGCGTGTGTGTGGCAGGGCTGAAGC
    ++:|:::||||||:||++|:++::|:||:++:||||||++||||||||:||||:||||||+
421 CGTTTTATTTTTTAGCGGTCGTTATTGTCGTTGTGTGCGTGTGTGTGGTAGGGTTGAAGC
                                                        MT461
481 GCCGCCCGCCTGGGTGCCAGGCGCGACTCGCCTCCCGGCATCGGGGGCTGCAGCGGGTGG
    +:+++::++::||||||||::|||++++|:|++::|::++|:||++||||:||:||++||||
481 GTCGTTCGTTTGGGTGTTAGGCGCGATTCGTTTTTCGGTATCGGGGGTTGTAGCGGGTGG

541 CTGCCTGGCGGTGGCCCGGGGCGGGGGTCGCGGGGCGCGGGCCGGCTGCGCGGTGGGGGT
    :||::|||++||||::++|||++|||||||++||||+++++||:++|:||++++|||||||
541 TTGTTTGGCGGTGGTTCGGGGCGGGGGTCGCGGGGCGCGGGTCGGTTGCGCGGTGGGGGT
        MC536
601 GGGGAGGGCCCCGCGGCCGGCGCGCGCCTCCACGCCGCGGGTTCTGCCGCAGCCTCTGGA
    ||||||||:::++++|:++|++++++::|::|++:++++|||:|||:++:||::|:||||
601 GGGGAGGGTTTCGCGGTCGGCGCGCGTTTTTACGTCGCGGGTTTTGTCGTAGTTTTTGGA
        MC593                                         MC650
661 AAATTAAACTTGCCGCTCTGATCCCGGCCGGGGCGGCGGCGGCGGCGGGCGGTGACCTTG
    ||||||||:|||:++:|:|||||::++|:++||||++|++|++|++|++||++|||::|||
661 AAATTAAATTTGTCGTTTTGATTTCGGTCGGGGCGGCGGCGGCGGCGGGCGGTGATTTTG

721 CGCGCGGGCGGGGGTCTCGGGGCCGCCCGGCCGCCCGCGCCCTCCCGCCGCCGCCGTCGC
    ++++++||++|||||:|++|||:++::++|:++::++++:::|::++:++:++:++|++:
721 CGCGCGGGCGGGGGTTTCGGGGTCGTTCGGTCGTTCGCGTTTTTTCGTCGTCGTCGTCGT
        MC727
781 CGCCGCCGCGCCCCTCCCCCCGCGCCCCGGCCGGCCGGCGGCTGTTTCCCGGGCGGCAGG
    ++:++:++++:::|:::::++++:::++|:++|:++|++|:|||||:::++||++|:|||
781 CGTCGTCGCGTTTTTTTTTTCGCGTTTCGGTCGGTCGGCGGTTGTTTTTCGGGCGGTAGG

841 CGGGCGGCGCGGTGCTCCTGTTGAATCCTCCCGCGGGCGGGCTTCTCCCCCTTCCGGGCG
    ++||++|+++++|||:|::||||||||||::|::++++||++||:|||:|:::::||:++|
841 CGGGCGGCGCGGTGTTTTTGTTGAATTTTTTCGCGGGCGGGTTTTTTTTTTTTTCGGGCG
        MC851
```

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Figure 2 Bisulfite conversion of the APRIN promoter-CpG island sequence. The top sequence represents the native sequence of the APRIN promoter. The bottom sequence shows the changes induced by the bisulfite conversion of the non-methylated cytidines. The conversion sequence was generated by the MethPrimer program that uses the assumption of full methylation in all the CpG units. The "+" signs indicate CpG units. The " | " signs indicate no change and the " : " signs indicate C to T conversion of the unmethylated C-s. The exon 1 sequence is boxed and is in blue. The primer sequences are in red and bold and their names are also indicated in red.

The individual projects from TASK 1 that were planned to carry on in the second year of the projects are as follows.

Project 6. Synthesize, optimize and evaluate various primer pairs for the targeted CpG sequences (from 3 post-operative samples and from cell lines LNCaP and MCF7) (Months 6-18).

Our promoter methylation experiments in the second year were performed using the primers we designed and tested in the first year. We generated three sets of primers. All of the primers are based on the highlighted areas in Figure 2, but they differ in some important details. The first set of primers (MX series) targets native DNA (the top strand sequence in Figure 2) and serves the purpose of the detection of native DNA to test yields and DNA quality after DNA isolations from minimal samples. The second set (MC series) detects methylated CpG units, while the third set (MT series) amplifies bisulfite-converted DNA for sequencing. The actual primer sequences are listed in Tables 1, 2 and 3 in the Progress Report for the first year.

Our experience for many months of work showed that most of the primer designs worked and the primers performed reliably. We had to redesign a few Methylation Specific PCR (MSP) primers, particularly the ones that contained uncertain (Y or R) base alternatives. We also learned that the efficiency of the MSP primers are sensitive for the presence of the Y or R base alternatives. Depending on the position of the uncertain base, the competition from the unstable variant affects PCR performance. Since PCR efficiency declines, some quantitative information is lost and that affects MSP-based methylation density estimations. We learned that for quantitative MSP studies by Real Time PCR we have to use primers without the ambiguous bases, or where the bases are very close to the 5' end of the primers.
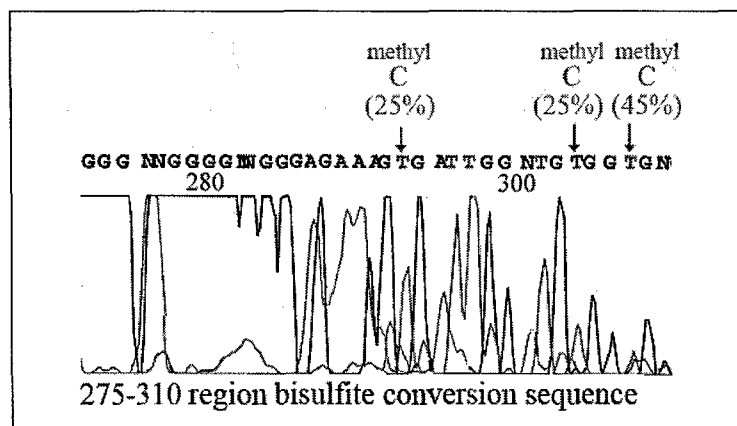
Project 7. Work out the best methods for methylation-specific PCR reactions and PCR product analysis (from 3 post-operative samples and from LNCaP and MCF7) (Months 6-18).

Another problem we realized is that using high quality, proof-reading PCR enzyme combinations for MSP is actually detrimental for amplifying bisulfite converted DNA. We noticed that if we resequenced the PCR products from an MSP generated by the TripleMaster kit (Eppendorf) or by the Extensor High Fidelity kit (ABGene), we found non-methylated bases (Ts) in the critical positions. MSP primers target methylated CpGs, where the mC is resistant to bisulfite, so the 3' base in the primer is C or G. If methylation is not 100 % in a particular CpG unit, the C converts to T. The primers, however, can also anneal to the non-methylated site, with a 3' mismatch. If we use a proof-reading PCR enzyme combination, the 3' exonuclease activity corrects the mismatch and we get a signal in non-methylated CpG sites. That explains our low success rate with the TripleMaster kit and with the Extensor kit. The PCRx Enhancer System with the Platinum Taq polymerase (Invitrogen) performed well overall. In most of these experiment we used easily available DNA samples from LNCaP and MCF7 cell lines.
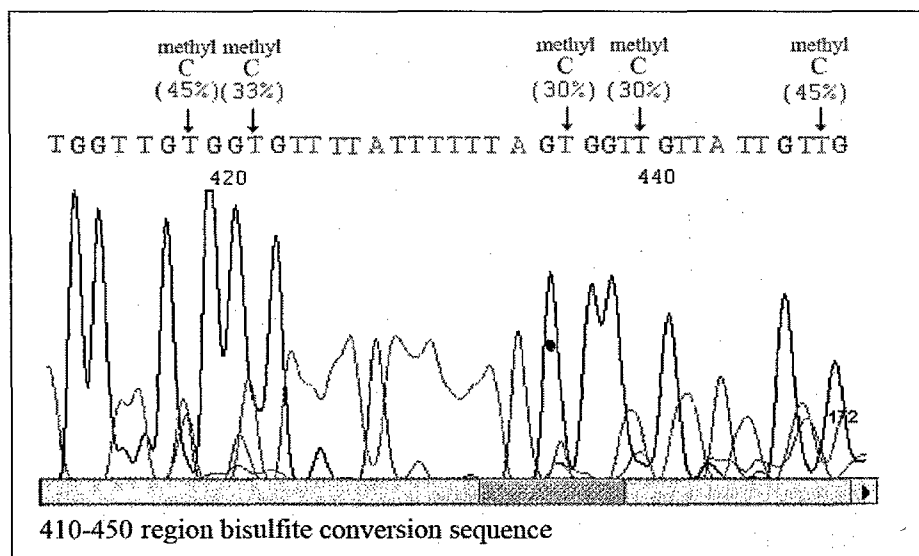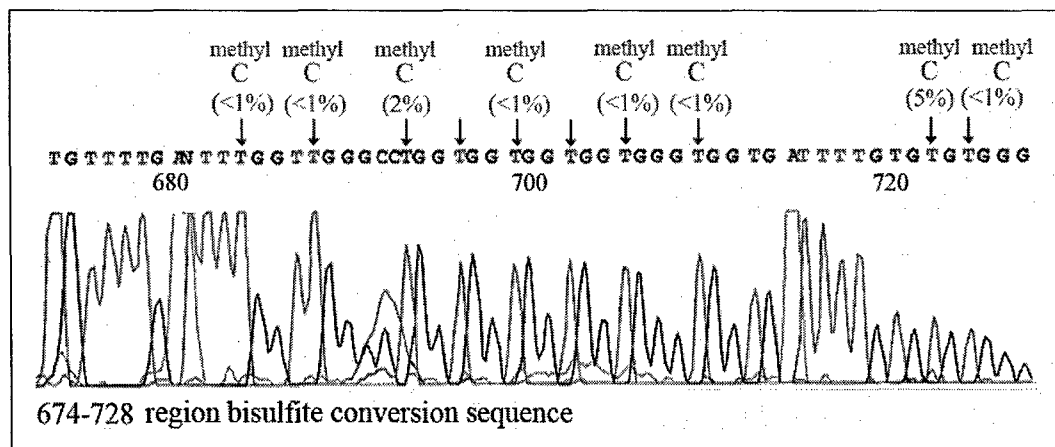
We had consistently positive methylation data by analyzing a prostate cancer cell line, the LNCaP variant LNCaP-TJA, a model for hormone resistant prostate cancer (12). Our preliminary data showed that the APRIN promoter in this cell line is methylated and silenced. It offered an excellent model to establish a biologically relevant methylation pattern that can be targeted by MSP in subsequent clinical studies.

We performed several MSP amplifications on bisulfite converted cellular DNA using the "MT" and "MC" series of primers (see above and in the first year Progress Report). We covered the entire CpG island around the 1st exon of APRIN, about 800 bp. The primers were selected to represent small, 100-150 bp units on average, keeping in mind that the DNA from future clinical, fixed samples may be fragmented. The amplicons with the expected sizes were purified and were sent for sequencing directly. We did not clone them on purpose, since we wanted to sequence the entire DNA populations from the amplicons. This approach can detect both the methylated and non-methylated portions of the CpG units. In this way not only the mapping of the methylated sites is possible, but we can also gain quantitative data on methylation density.

In the next section, **Figures 3-7** will show representative results of our sequencing efforts in panels of colored graph format. We kept the original printouts but added the base-positions (in reference to Figure 2) at the bottom and indicated the methylated cytidine positions and ratios in percentage above the sequences. (The position 78-133 area has been presented in Figure 9 of the first progress report)

Figure 3. Methylation sequencing in positions 1-65 after bisulfite conversion.

1-65 region bisulfite conversion sequence

Figure 4. Methylation sequencing in positions 158-240 after bisulfite conversion.

158-240 region bisulfite conversion sequence

Figure 5. Methylation sequencing in positions 275-310 after bisulfite conversion.

275-310 region bisulfite conversion sequence

Figure 6. Methylation sequencing in positions 410-450 after bisulfite conversion.

410-450 region bisulfite conversion sequence

Figure 7. Methylation sequencing in positions 674-728 after bisulfite conversion.

674-728 region bisulfite conversion sequence

The data clearly show that the APRIN promoter is methylated, but methylation is not uniform and not complete in the TJA cell line. The integrated methylation map and methylation density data are demonstrated in **Figure 8.**

Figure 8.   Comparison of the integrated CpG and methylation maps and the integrated CpG and methylation density maps.
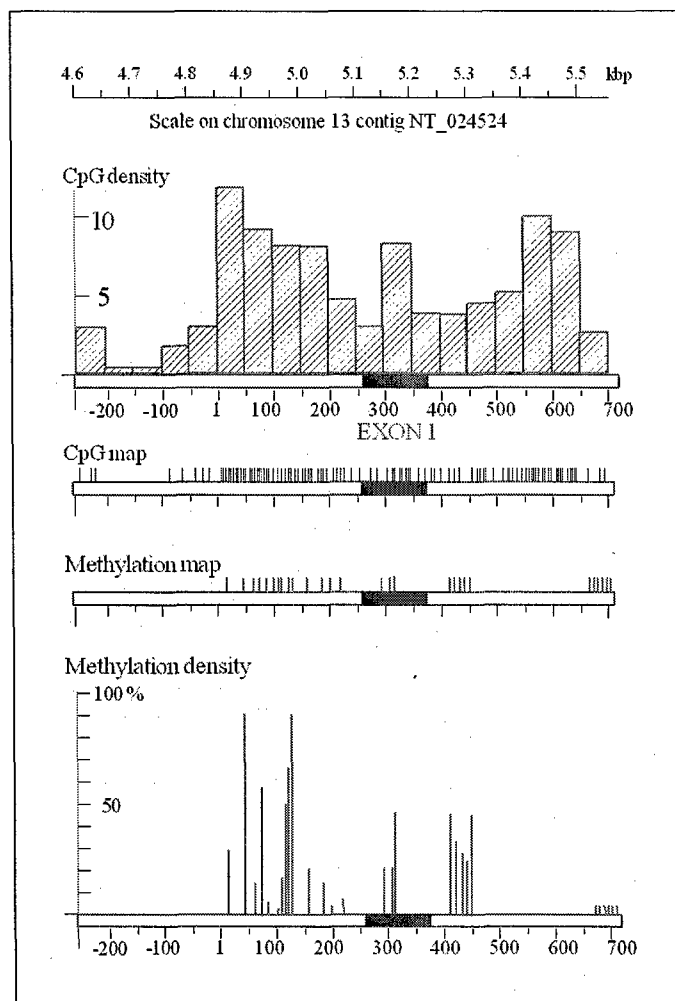
Figure 8 legend. The CpG density panel and the CpG map are from Figure 1. The methylation density and methylation map data are shown on the same scale as those of the CpG data. Methylation density is displayed in percentage of the total as indicated at the left.

The above global methylation data are instructive in several aspects. Although the overall methylation pattern follows CpG density, there are differences. Most importantly, the 100 bp area immediately upstream of Exon 1 has low level methylation. This site is involved in the assembly of the transcription complex, it is high in CpGs and the lack of methylation is surprising in terms of silencing. The high level of methylation in the 50-150 area, which also coincides with the peak of CpG density, may indicate a critical regulatory element. We could not detect methylation in the position around 600, in contrast to the second highest CpG density peak there. The data indicate the non-random nature of methylation and that the positions of the methylated CpGs are critical.

The most important impact of our methylation sequencing results is that it identified methylation hot spots in the APRIN promoter CpG island, e.g. the 100-160 CpG positions, the 295-307 positions and the 420-450 positions. We focused our attention on the 100-160 position, for its highest levels of methylation, its coincidence with the CpG density maximum and as the most probable site to interfere with transcriptional activity.

Project 9.   Preliminary screening for informative methylated CpG targeting primers: characterize the range of methylation (methylation coefficient) in a small sample for each successful methylated CpG PCR target (using the above 10 postoperative samples, 20 archive samples and 10 needle biopsy samples, tumor and normal). (Months 6-24)   The details of this project will be discussed together with TASK 2.

Project 10. Establish computer registry and record maintenance of samples (Months 1-24).
We have a small number of clinical samples from our local clinical resources. These samples are recorded and stored in a de-identified system, according to the regulations by the DOD and by the Tufts University Institutional Review Board. The other sources of our clinical samples, the commercially available prostate cancer tissue arrays are exempt materials. They were purchased with a commercial, de-identified record of histological diagnosis and staging.

TASK 2: *To perform low- to medium-scale analyses of clinical samples to gain preliminary data on the correlation between promoter methylation and prostate pathological conditions. (continuous activity in the second year) (Month 12-24)*

Project 1. By using the established tissue- and DNA-bank samples and newly collected material (40 patients, needle biopsy), the selected highly informative methylation markers will be used to analyze epigenetic gene silencing in various prostate pathological conditions (Months 12-24).

To study methylation in the APRIN promoter from clinical samples, first we used commercially available tissue samples from tissue array preparations. These de-identified and exempt commercial products contain sample sections from 20 to 70 patients with histological diagnoses from expert pathologists. We purchased the tissue arrays from the Cybrdi Company (Catalog number CC19-01-003). To extract the DNA from the tissue sample, we used the EX-WAX DNA Extraction Kit from the Chemicon company, following the instructions for the kit.

We tested the DNA preparations for DNA content and quality, particularly in the target APRIN promoter area. Based on our experience, the Mx400-Mx461 primer pair is sensitive for amplifying native DNA. We could detect the first intron sequence from the APRIN gene in most of the preparations as shown in a representative assay in **Figure 9.**

Figure 9. PCR analysis of native DNA preparations from prostate cancer tissue array samples.
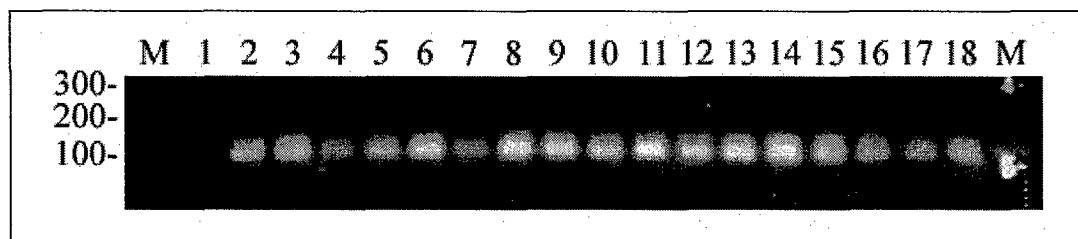
Figure 9 Legend. Agarose gel assay of PCR amplicons from native DNA preps from prostate cancer tissue samples. We used the Mx400-MX461 primers, the PCRx Enhancer System with the Platinum Taq polymerase in conditions described in the first year Progress Report. M, molecular size marker, the sizes are at the left in base pairs. Number 1 is no-DNA control; numbers 2-18 indicate sample DNA preparations.

We detected amplifiable DNA, the starting material to our methylation studies. Next these DNA preps were used for methylation analysis by methylation specific PCR. After bisulfite conversion, we tried several primer pairs targeting the methylation hot spot areas we identified. First we show the successful combination that targets the position 133-229 area. The MSP results by using the MC133-MC229 primer pair are shown in **Figure 10.**
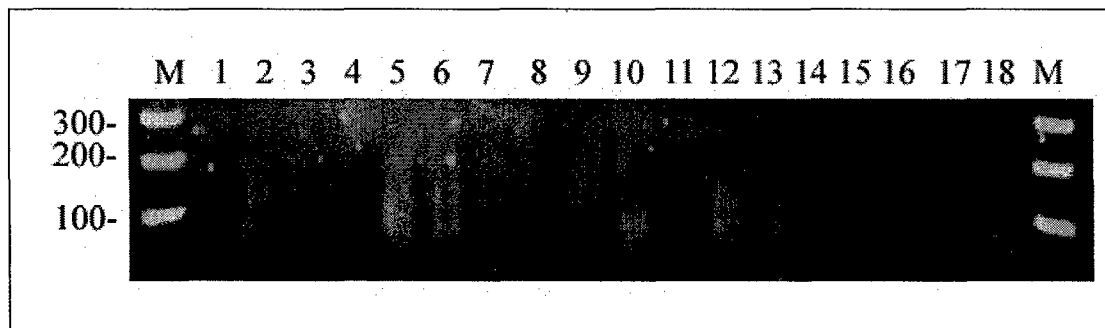
Figure 10. Methylation specific PCR from prostate cancer samples, targeting the position 133 promoter area.
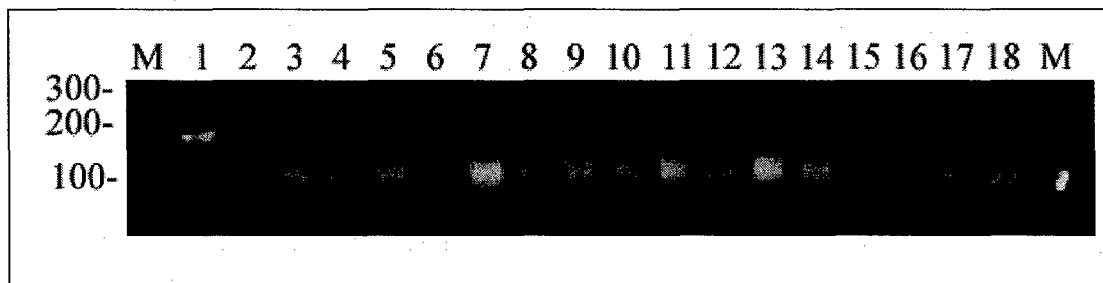
Figure 9 Legend. Agarose gel assay of MSP amplicons from bisulfite-converted DNA from prostate cancer tissue samples. We used the MC133-MC229 primers in the PCRx Enhancer System with the Platinum Taq polymerase. M, molecular size marker, the sizes are at the left in base pairs. Number 1, TJA DNA control with the MC50-MC133 primer pair (positive control, a PCR reaction control); numbers 2-18 indicate the same preparations shown in Figure 9. The histological grading of the samples is the following: sample 2, benign prostatic hypertrophy; samples 3-6, adenocarcinoma, grade I; samples 7-10, adenocarcinoma, grade II; samples 11-14, adenocarcinoma, grade III; samples 15-18, undifferentiated adenocarcinoma.

Further efforts to detect methylation by MSP using other primers met with limited success. Our efforts to detect methylation by the MC50-MC133 primers resulted in weak signals in smeary backgrounds, as shown in **Figure 11.** The results were similar from other DNA preparations, so we suspect some inhibitor in the tissue array, or covalently bound fixation artifacts in the particular promoter area blocked the polymerase or primer annealing.

Figure 11. Methylation specific PCR from prostate cancer samples, targeting the position 50-133 promoter area.

Figure 11 Legend. Agarose gel assay of MSP amplicons using the MC50-MC133 primers on bisulfite-converted DNA from prostate cancer tissue arrays. The sample order, the abbreviations and the numbers are the same as in Figure 10.

To analyze if the amplification problem was primer related, we also tried a primer pair that differs only in minor details, the MT series. These primers were designed to amplify bisulfite converted DNA, but instead of MSP, they were designed for sequencing. The MT50-MT133 primers were even less successful as shown in **Figure 12.**

Figure 12. Methylation specific PCR from prostate cancer samples, targeting the position 50-133 promoter area with the MT series primers.
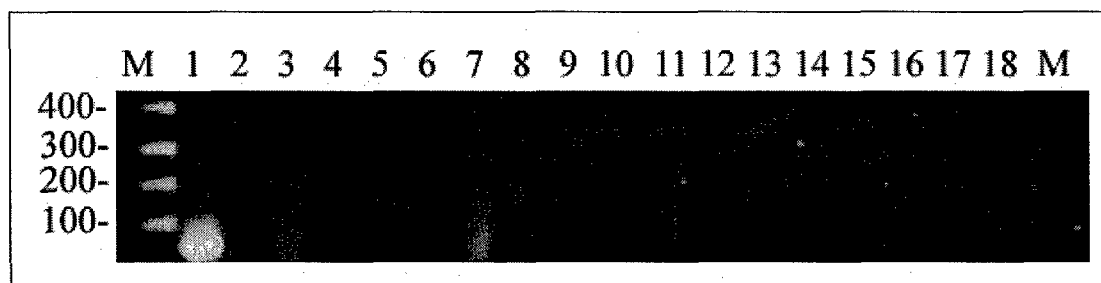
Figure 11 Legend. Agarose gel assay of MSP amplicons using the MT50-MT133 primers on bisulfite-converted DNA from prostate cancer tissue arrays. The sample order, the abbreviations and the numbers are the same as in Figure 10.

The results indicate that we found the experimental conditions to detect methylation in the APRIN promoter from clinical samples. Our problems with the important promoter methylation hot spot, the 133 area need to be addressed in future studies. We plan to change the DNA isolation method, we will try to do antigen-retrieval technology that works in immunohistochemistry and we will try other primer designs.

Overall, however, we were able to establish some degree of preliminary correlations with the methylation state of the APRIN promoter and the histological grading of prostate cancer. The average signal intensity in Figure 10 shows the tendency to correlate with grading. It is very difficult to normalize our data at this stage. The preparations contain very little DNA, the only information is the native PCR in Figure 9. After normalization based on Figure 9, the correlation disappears, but the results strongly suggest the lack of methylation in BPH and the presence of methylation as early as in grade I. We analyzed 33 individual patient samples, but the results are inconclusive. Further efforts are needed and an increase in sample numbers to investigate if methylation correlates with cancer pathology.

To increase the sample numbers, we also analyzed a series of prostate cancer specimens from our collaborator's tissue bank. We received laser caption microdissected (LCM) samples representing normal periferal zone, benign prostatic hypertrophy (BPH) stroma and gland samples, moderate prostatic intraepithelial neoplasma (PIN) samples, carcinoma samples from grade III, grade IV and intraductal carcinoma samples. To extract DNA from the samples we used the PicoPure DNA Extraction Kit from Arcturus and the DNeasy Tissue Kit from Qiagen. We tested the DNA preparations for DNA quality, using the Mx400-MX461 primer pair. We could detect the first APRIN intron in the native DNA in half of the preparations as shown in **Figure 12.**
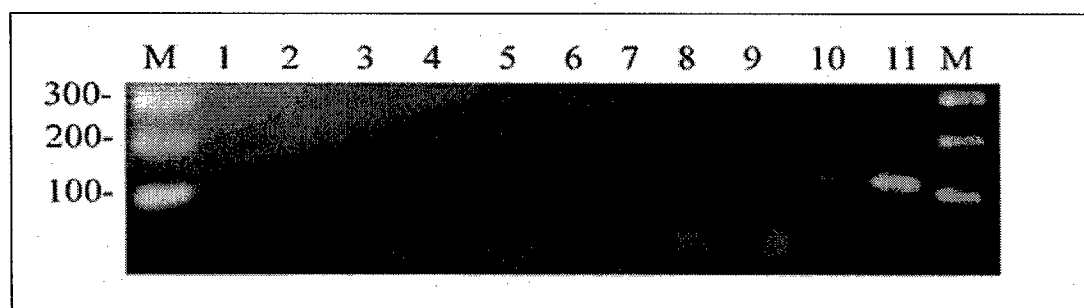
Figure 12 Legend. Agarose gel assay of PCR amplicons from native DNA preparations by laser capture microdissection from prostate cancer samples. We used the Mx400-Mx461 primers, the PCRx Enhancer System with the Platinum Taq polymerase in conditions described in Figure 9. M, molecular size marker, the sizes are at the left in base pairs. Number 1 is no-DNA control; numbers 2-11 indicate sample DNA preparations.

Figure 12. PCR analysis of native DNA preparations (LCM) from prostate cancer samples.
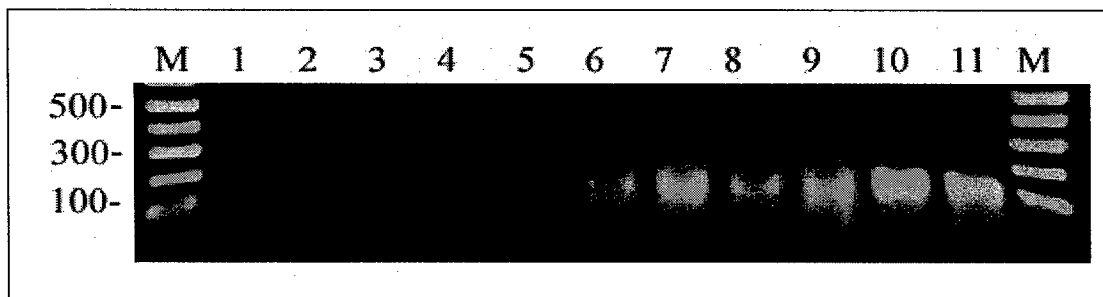
After bisulfite conversion, we performed MSP by using the MC133-MC229 primer pair. The result is shown in **Figure 13.**

Figure 13. Methylation specific PCR from laser capture microdissected prostate cancer samples using the MC133-MC229 primer pair.

Figure 13 Legend. Agarose gel assay of MSP amplicons using the MC133-MC229 primers. Experimental conditions were the same as in Figure 10, Number 1, no-DNA control; numbers 2-18 indicate the same preparations shown in Figure 9. The histological grading of the samples is the following: sample 2-3, normal control; samples 4-6, benign prostatic hypertrophy; samples 7-9, PIN lesion; sample 10, adenocarcinoma, grade III; sample 11, adenocarcinoma, grade IV.

We could detect promoter methylation only in grade III and grade IV prostate cancer samples in the laser captured samples. The fact, however, that we lost half of the DNA samples may indicate DNA degradation problems in our other samples. So, although it appears again that there is correlation with methylation and cancer grading, technical problems made the results inconclusive. Our future efforts will include further optimization in DNA isolation techniques and to increase prostate cancer samples.

Our access to fresh clinical samples were very limited in the second year of the project. Our collaborator at the Urologic Oncology Department of the Tufts-New England Medical Center, Dr John Long left the institution. We succeeded in finding another surgical expert at the same institution, Dr Gennaro A. Carpinito. Since we changed a key collaborator, both the local IRB and the Human Subjects Research Review Board of the US Army Medical Research and Materiel Command required us to re-certificate our human material projects. The procedure was already in progress when the local IRB changed its policy on investigator qualifications and Dr Carpinito was required to take additional exams. All this took an enormous time and in the meantime we had no access for clinical samples.

By the beginning of this year it became obvious that we could not finish this part of the project in a timely fashion, for reasons beyond our control. We communicated this with the US Army Medical Research and Materiel Command and we were granted a one year no cost extension to complete the project.

At the same time we began exploring alternative avenues and resources to investigate human prostate samples. One option is the NCI Cooperative Prostate Cancer Tissue Resource. For a fee and after a certification procedure, fresh frozen and paraffin embedded prostate cancer samples are available with well characterized pathological background. The second option is the National Disease Research Interchange resource, funded by the National Institute of Health. After an approval procedure, a variety of frozen and fixed tissue samples are available. The third option is the Asterand company that sells human tissue specimens, tissue arrays and cell lines. Regardless of when we will be cleared to have access for postoperative human samples within the university, the above options appear to be simpler and we have already started the application procedures for the NCI and NDRI resources.

Project 2. Perform feasibility experiments using a set of well established new markers to detect promoter methylation from circulating cancer cells from blood (native or enriched) (from 20 samples of 20 patients of the above 40) (Months 12-24). See our comments in Project 1. This project will be performed in the third year extension period.

Project3. Data analyses to establish the first correlations between methylation driven AS3 promoter silencing and various prostate cancer conditions (Months 18-24). For preliminary data, see our results in Project 1.

Project 4. Compare AS3 promoter methylation changes with other clinical markers of prostate cancer risk, such as the prostate specific antigen (PSA) (Months 18-24). See our comments in Project 1. This project will be performed in the third year extension period.

KEY RESEARCH ACCOMPLISHMENTS

1) Further optimized and established novel primer design principles for applications in methylation specific PCR.

2) Refined the conditions and methodology for methylation sensitive PCR and sequencing.

3) Analyzed the entire CpG island in the APRIN promoter area for DNA methylation. Methylation specific sequencing generated methylation data in the 800 bp region.

4) Established the methylation map and methylation density in the APRIN promoter in a hormone resistant cancer cell line. The LNCaP-TJA cell line is a model for hormone resistant prostate cancer and can not differentiate. APRIN, a differentiation factor in these cells is silenced and our data indicate promoter methylation mechanisms. Our methylation map identified biologically relevant methylation hot spots in the promoter.

5) By targeting the identified hot spots, we performed methylation analyses on clinical prostate cancer samples. We did not find methylation in benign prostatic hypertrophy, but we found that low level methylation occurs early in grade I cancers. We saw indications that the methylation status in the APRIN promoter correlates with histological grade, but these data are only preliminary

6) Optimized the methodology to detect promoter methylation from minute clinical samples and established the first working epigenetic markers for APRIN silencing in prostate cancer.

REPORTABLE OUTCOMES

Peter Geck, Yuping Xia. (2005) Promoter methylation silencing of the APRIN gene in breast, prostate and ovarian cancers. Proc Amer Assoc Cancer Res 2005, 46:921

Geck, P., Maffini, M. and Shu Ling Liang (2004) The APRIN gene is downregulated in invasive cancers. Proc. Am. Assoc. Cancer Res. 45:1292
> (Oral presentation in the 95th Annual Meeting of the American Association for Cancer Research, 2004, March 27-31, Orlando, FL.)

Grant award based in part on data generated by this project.
> 2004-2007 Grant award from The Susan G. Komen Breast Cancer Foundation, "Does BRCA2 Justify prophylactic mastectomy? Allele specific methylation markers of BRCA2 v. AS3 losses in breast cancer" BCTR0403214, Principal Investigator, $250,000

CONCLUSIONS

Based on our results in the first year of the project, we established further important milestones in the second year. At the technical level, we optimized our primer design and methylation specific PCR and sequencing methodologies.

We established the entire methylation pattern and methylation density data on the APRIN promoter in a hormone resistant prostate cancer model. These data established that the methylation pattern is selective and regulated (non-random), and identified biologically relevant methylation hot-spots in the promoter of the APRIN gene.

We used the methylation hot spot information and performed methylation studies in clinical prostate cancer samples. We detected methylation in the APRIN promoter and we found preliminary correlations between methylation and histological grading.

Overall, we achieved further significant progress towards our goals. We established the first epigenetic markers to study APRIN silencing in prostate cancer. These novel reagents will allow us to study the correlations between epigenetic regulation and prostate cancer biology and to develop an early diagnostic and prognostic marker system in the clinical management of prostate cancer. This will be our major goal in the third year of the project by using clinical resources within our institution and outside (NCI, NDRI, Asterand, etc.)

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